Determination of Molecular Weights by Sedimentation Equilibrium

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Sedimentation equilibrium provides a powerful method for determination of the quaternary structure of associating macromolecules, as well as for study of the stoichiometry of heterogeneous binding reactions. Both types of study rely upon a determination of the average molecular weights of species allowed to equilibrate in the sample cell of the analytical ultracentrifuge rotor. In order to better understand the analyses employed to study associating systems, it is useful to examine the methods used for simple determinations of molecular weights. Although several methods exist for obtaining molecular weights that appear to be simpler and less time consuming, the versatility and power of sedimentation analysis endows the analytical ultracentrifuge with a unique position in the array of alternative techniques.

With respect to molecular weight determinations, the advantages of sedimentation analysis are as follows. The wide range of rotor speeds makes possible the study of molecules as small as sucrose and as large as a virus of 50 million daltons. For polydisperse samples, average molecular weights can be obtained as well as some measure of the molecular weight distribution. The usual methods employed do not require any assumptions about shape or hydration of the solute under study. Furthermore, the measurements made in the ultracentrifuge are primary. Unlike commonly used methods that require the use of standards, no calibration with materials of known molecular weight are required. This is particularly important when the molecule being analyzed and the standard molecules behave somewhat differently due to subtle differences in physical parameters.

Two general methods for determining molecular weights using analytical ultracentrifugation have been used: the sedimentation and diffusion method and the sedimentation equilibrium method. The first of these, widely used in the past, has been largely replaced by the sedimentation equilibrium method. It is of historic interest, however, and will be covered in a brief section at the end.

The most common method of molecular weight determination is sedimentation equilibrium. This technique depends on the measurement of the solute concentration distribution in the sample cell when equilibrium conditions have been attained, that is, when the distribution of solute due to sedimentation is balanced by the distribution due to diffusion.

While the theory of sedimentation equilibrium can be viewed from a kinetic standpoint[1] or from a thermodynamic one,[2] the equations relating the final concentration distribution in the cell to the molecular weight of the solute are the same in both cases. In the kinetic treatment, the equilibrium condition is seen to be achieved when the rate of transport of material in one direction due to sedimentation is exactly balanced by the rate of transport in the other direction due to diffusion. In the thermodynamic treatment, equilibrium is reached when the total potential (the sum of the chemical potential and the centrifugal potential) is independent of time and of position in the cell. Strictly speaking, the final equilibrium condition is never actually attained because it would require infinite time. In practice, it is possible to achieve in a reasonable time interval a condition that is, for computational purposes, equivalent to the theoretical equilibrium condition. This practical equilibrium condition is one in which the concentration distribution in the centrifuge cell does not change with time within the experimental error in measuring the distribution.

Compared to the sedimentation and diffusion method mentioned above, the equilibrium technique has several advantages: (a) it has a secure theoretical basis in thermodynamics so that fewer assumptions are required in order to apply the method to experimental data, (b) it is simpler experimentally, and (c) it is more accurate. Sedimentation equilibrium is especially preferred over sedimentation and diffusion in studies of polydisperse solutes, or for solutions containing high concentrations of salts or denaturing agents.

Conventional Sedimentation Equilibrium Method

"Conventional" is used here to distinguish this method from approach-to-equilibrium, short column, and meniscus depletion methods. Conventional sedimentation equilibrium experiments are defined as those experiments that utilize a fluid column length of 3 to 5 mm in the centrifuge cell, with rotor speeds sufficiently low so that solute concentration at the meniscus is not zero, and with the entire contents of the cell at sedimentation equilibrium. In order to compute molecular weights, the concentration of the solute throughout the cell must be determined when equilibrium is reached. It is therefore important to know at least an approximate time to reach equilibrium (the transient time), and an appropriate rotor speed. If an approximate molecular weight for the solute is known, then operating speeds and transient times can be estimated from equations available in the literature.[2, 3] However, the final judgment as to when equilibrium has been reached is based on experimental factors: once presumed equilibrium has been reached, take scans several hours apart at intervals of approximately one-fifth the estimated equilibrium time and compare the data. When the difference from scan to scan is the same as the noise in the data, assume equilibrium has been reached.

The criterion usually given for the optimum rotor speed for 3-mm solution columns is one that will result in a solute concentration at the bottom of the cell that is four times the concentration at the meniscus. This rule is applicable only for homogeneous, ideal solutes and should be considered to be a minimum speed. Figure 1 can be used to obtain an estimate of an appropriate operating speed if an approximate molecular weight is known. If no information about the sample is available, make a separate sedimentation velocity run[4] and determine an approximate sedimentation coefficient; then use Figure 1. This first estimate of operating speed can be off as much as 50% without any serious consequences.

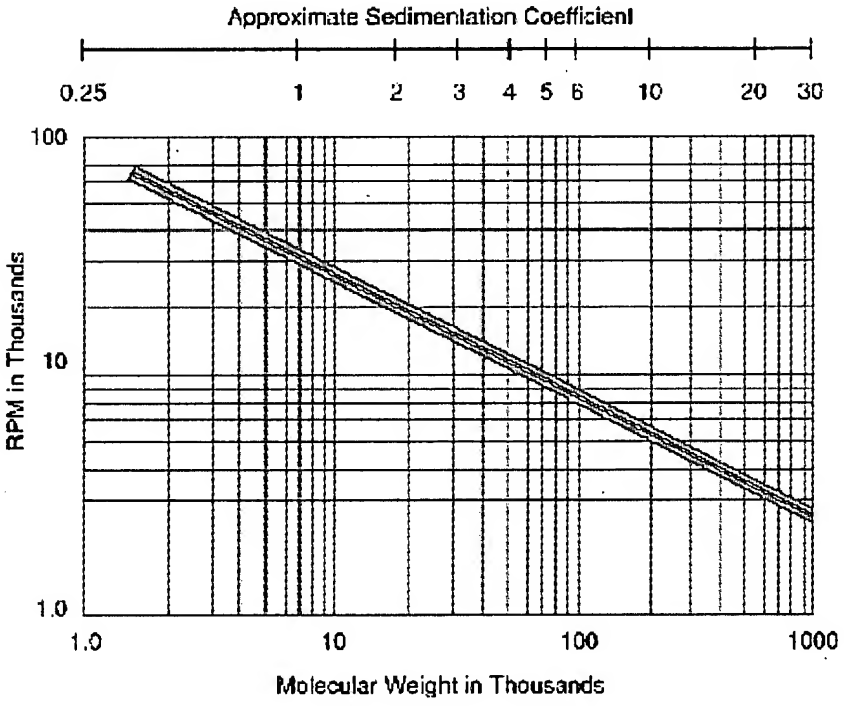

Figure 1. Nomogram for selecting an operating speed for a sedimentation equilibrium run.

Transient time can be reduced by a technique known as overspeeding.[5, 6] This is a simple procedure in which a rotor is initially run at a speed higher than the equilibrium speed to promote rapid redistribution of the solute. The rotor speed is then decreased to the selected speed and the solute is allowed to reach its final equilibrium distribution. During the initial period, bulk solute is transported by sedimentation toward the bottom of the cell, so that the quantity of material in the upper and lower halves of the cell grossly approximates that required for the final equilibrium condition. The rotor is then decelerated to a speed lower than the equilibrium speed to allow diffusion of the steep gradients formed during the initial period. Finally the rotor is accelerated to the equilibrium speed and allowed to run until the required

solute distribution is attained. A detailed procedure for computing the required speeds and times of operation for each step has been described by Hexford, Radford, and Beams.[5] In practice, two speeds are adequate: an initial overspeeding period followed by reduction to the equilibrium speed. Although this is a valuable technique, it is not without a hazard: if the concentration of the solute at the bottom of the cell becomes so high that it forms a gel-like precipitate, it may not redissolve when the speed is reduced. The following method is the best general procedure for most samples. The fluid column is sufficiently short so that the time required for attainment of the equilibrium condition is not excessive (most proteins will require 24 hours or less). At the same time, the column length is sufficiently long to allow accurate determination of molecular weights and assessments of possible heterogeneity or nonideality. If the sample is polydisperse, then the point weight-average molecular weights across the cell, as well as the weight-average and *z*-average molecular weights for the whole sample can be calculated from the same experimental data. [note 1] For reacting systems, the same procedure can be used and extended to approximate the equilibrium constants for the system. For studies of associating systems, longer column heights are preferred because the greater amount of data obtained will result in higher precision.

Recommended Procedure

The best general references are Richards and Schachman,[7] LaBar and Baldwin,[8] and Van Holde.[3]

Sample and cell: use a 12-mm double-sector cell, quartz window, and a double-sector centerpiece. Torque to 120 inch-pounds. With the screw-ring end facing you and with the filling holes up, place 110 ?L of sample diluted in buffer or salt solution[9] in the right-hand sector of the cell. Absorbance of the sample solution should be close to 0.2 to 0.4 at the selected wavelength. In the left-hand sector, place 120 ?L of solvent, preferably the dialyzate of the sample solution. Adjust the weight of the appropriate counterbalance to within 0.5 g of the weight of the filled cell, *but not more than the weight of the cell.* Align the cell and counterbalance carefully.

Optionally, FC-43[note 2] oil can be placed in the bottom of double-sector centerpieces. In addition to lifting the aqueous sample solution off the cell bottom (necessary for some centerpieces), FC-43 has the advantage of a refractive index very close to that of water. Consequently, FC-43 won't reflect light at the cell bottom to the same extent that the centerpiece material will, and data collected in this region may be more reproducible. Place 30 ?L of FC-43 in the right-hand sector of the cell followed by 80 ?L of sample in dilute buffer or salt solution. (The FC-43 may contain impurities that cause the sample to aggregate, in which case it can be omitted.)

If you choose to overspeed to reduce the time to equilibrium, initially use a speed 1.5 times the final equilibrium speed (but not in excess of the speed limit of the rotor) and run at that speed for about one-fifth of the total estimated equilibrium time. Decrease to the final equilibrium speed and run at that speed for the remainder of the estimated transient time. When equilibrium has been reached (see above), stop the run and analyze the data.

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[1] A weight-average molecular weight is defined as $M_w = \Sigma C_i M_i / \Sigma C_i$ and a *z*-average molecular weight is defined as $M_z = \Sigma C_i M_i^2 / \Sigma C_i M_i$, where C_i is the concentration of component *i*, and M_i is the molecular weight of component *i*.

[2] FC-43 oil (3M Company) is a dense (1.92 g/mL), immiscible fluorocarbon.[10]

Data Analysis

For any macrosolute with negligible nonideality, the concentration distribution in the centrifuge cell at equilibrium is described by

$$C_t = C_{r_0}\, e^{[M(1-\bar{v}\rho)\,\omega^2/2RT)]\ (r^2-r_0^2)}$$

where

C_r	=	concentration of macrosolute at any radial distance
C_{r0}	=	concentration of macrosolute at the reference radial distance r_0
omega	=	rotational velocity in radians/s
v-bar	=	partial specific volume of macrosolute
rho	=	density of solution
R	=	gas constant
T	=	absolute temperature
M	=	molecular weight

(In practice, this equation is often written with absorbance, *A*, substituted for concentration, *C*, assuming by Beer's law that absorbance is proportional to concentration.) Rearranging the equation yields a way to calculate molecular weight:

$$\frac{d\ln(C_r)}{dr^2} = \frac{M(1-\bar{v}\rho)\omega^2}{2RT}$$

Plotting $\ln(C_r)$ versus r^2 will give a line with a slope that equals M(1 - v-bar rho), the buoyant molecular weight of the macrosolute.

Complications arise, however, when this plot is not a straight line. A downward curving plot, indicating that the molecular weight decreases with increasing concentration, is an indication that the solution is nonideal, and that the molecular weight exhibits concentration dependence. An upward curving plot, on the other hand, is an indication that the macrosolute is polydisperse. It is a mixture of molecular weights either because it is impure or because sample material has aggregated. In this case $d\ln(C_r)/dr^2$ yields an average molecular weight that, providing all macrosolute species have the same partial specific volume, gives the weight-average molecular weight, M_w.

If the polydispersity is a reflection only of a self-associating system where aggregates are in reversible equilibrium with monomers, a weight-average molecular weight at any point in the cell will depend only on the concentration of the macrosolute at that point. Plots of M_w versus solute concentration, when runs are done at different rotor speeds, should all overlap within experimental errors. If, on the other hand, the polydispersity is not due to a self-association, plots of M_w and C_r at different rotor speeds will not overlap.

Using the linearity of the $\ln(C_r)$ and r^2 plot as an assessment of homogeneity is not without pitfalls. One is the inability of the eye to detect minor deviations. For example, the curvature caused by dimerization of as much as 10% of the solute will generally be undetectable. A more subtle effect exists when a sample exhibits both concentration dependence and polydispersity. One may cancel out the other, and

the plot of $\ln(C_r)$ and r^2;would be a straight line, although the sample is neither homogeneous nor ideal. Experiments at other concentrations and/or other rotor speeds will provide a test for both effects. At higher speeds the effects of heterogeneity should become more obvious. At lower concentrations the effects of nonideality will decrease in an almost linear fashion. If the sample is known to be homogeneous by some other means, then a curving plot will be due only to nonideality. In general, if the plot is a straight line and the speed sufficient to assure a large change in concentration from the top to the bottom of the cell (C_r/C_{r0} of 4 to 10, for example), then one can be reasonably sure that the molecular weight value obtained is independent of concentration.

While the plot of log (C_r) versus r^2 is a well-established approach to the analysis of sedimentation equilibrium data, there are limitations, as illustrated above, to this method of analysis and one may wish to use a more sophisticated approach to data analysis.

Molecular Weights from Sedimentation and Diffusion Data

The sedimentation and diffusion method (s/D) is based on the relationship between the sedimentation coefficient and the molecular weight of the molecule. The well-known Svedberg equation[2, 10] is used for this calculation.

$$M = (RTs/D)(1 - \bar{v}\rho)$$

The inherent limitation of this method of determining molecular weights is the need to know not only the partial specific volume of the solute and the density of the solution, but also the sedimentation and diffusion coefficients of the solute. These values, determined at a single finite concentration, can be used for the computation of an apparent molecular weight value providing that the experimental conditions of solvent, concentration, and temperature are identical for both determinations. If *s* and *D* values are used, then theoretically the value of the density used in the equation should be the density of the sample solution in water at the experimental concentration, at 20?C. In practice, the value for the density of water gives results of sufficient accuracy. The value for *T* used in this case is 293.2?K.

The best results are obtained using values of *s* and *D* that have been determined at a series of concentrations in the same solvent at the same temperature and extrapolated to zero concentration. These values of *s* and *D* (not necessarily corrected to water at 20?C) are then used in the Svedberg equation, along with the density of the solvent and the experimental temperature. The value of the molecular weight obtained by this method is that of the anhydrous molecule, providing the v-bar used is the anhydrous value.

Compared to the sedimentation equilibrium technique, the *s/D* method has an advantage when paucidisperse solutes are studied because molecular weights can be determined for each component separable by sedimentation velocity (providing the essential diffusion data are available). The same advantage applies in special cases of mixtures of molecules of the same size and different shape or of the same shape and different size. In the study of some molecules that are too large for the sedimentation equilibrium technique, the s/D technique may be used advantageously. In particular, large nucleic acids cannot be studied by sedimentation equilibrium methods, so molecular weight estimations for these materials are generally made based on sedimentation coefficients.

When a macromolecule, which has been shown to be pure by other methods, demonstrates heterogeneity with respect to mass by sedimentation equilibrium, further experiments are needed to distinguish a self-associating system from one where the polydispersity is due to contaminants. For associating systems, the monomer molecular weight as well as the degree of aggregation can be estimated. Consult the literature as suggested below for further uses of the sedimentation equilibrium technique:

- Computation of point average molecular weights[11]
- Estimation of dissociation constants for reacting systems[12, 13]
- Study of molecular weight distributions, including synthetic polymers in organic systems[14-20]

In addition to the conventional approach described here, variations such as approach-to-equilibrium[4, 21-24] and the short column technique[14, 25-27] provide, for appropriate samples, the power of the

traditional method with the advantage of reduced run times.

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